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SCREENING METHODS FOR TOXIC SUBSTANCES

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The objective of this project was to explore the feasibility of using fluorescent probes as a technique for detecting effects produced by the action of neurotoxic chemicals. This approach may ultimately provide a more sensitive test for adverse biological effects needed in the hazard evaluation of agricultural and other chemicals.

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Director,
Health Effects Research Laboratory

ABSTRACT

The present report deals with the application of fluorescent probe technique to the detection of conformational changes in rat brain synaptosomes under the influence of certain neurotoxic chemicals, chlorophenothane (DDT) and diphenylhydantoin, which are expected to produce functional changes in nerve membranes. Pretreatment of the rats with (i) a single dose of DDT capable of producing neurological symptoms and (ii) repeated sub-lethal doses of DDT did not alter the fluorescence characteristics of the hydrophobic probe 8-anilino-1-naphthalene sulfonic acid (ANS) bound to the synaptosomes. DDT treatment also showed no effect on native protein fluorescence of synaptosomes.

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SECTION 1

INTRODUCTION

GENERAL

A large number of industrial and agricultural chemicals including pesticides, detergents, solvents, plastics, chemical intermediates, and plasticizers may be introduced into the environment as effluents from manufacturing plants, or during their use and disposal. Among the major concerns associated with the presence of these chemicals in the environment is their potential toxicity to man. From a toxicological standpoint, the nervous system is one of the more sensitive sites of action of various environmental contaminants.

At present, a toxicological evaluation of neurotoxic chemicals usually consists of measuring a given organism's behavioral response, neurophysiological activity and examining pathological changes in the nervous system. These methods are suitable for detecting neurotoxic disorders resulting from acute or chronic toxicity, but may not be able to detect subtle effects or a minimal degree of damage resulting from low-level exposure to these chemicals. Evidence has been presented which shows that subclinical nerve damage was detected in workers exposed to lead who showed no clinical neurological symptoms.[1,2] Therefore, reliable, sensitive techniques are needed to detect early damage resulting from a variety of neurotoxic substances.

Although various neurotoxic substances like DDT, nicotine, pyrethroids, tetrodotoxin and methylmercury act via different mechanisms, an effect upon the nerve membrane as a whole is implicated with all of them.[3,4,5,6,7] It is generally believed that these chemicals interact specifically or non-specifically with components of the nerve membrane, thereby leading to possible changes in membrane function. Such changes may account for the neurophysiological effects of these chemicals. It is conceivable that such alterations may be preceded by changes in the conformation or structural organization of the nerve membrane. The conformational changes could therefore be used as an indicator of neurotoxicity.

In recent years, fluorescent probes have been employed for detecting structural or conformational changes in membranes and other biological structures.[8,9,10,11,12,13] The high sensitivity of fluorescent probes to their microenvironment permits one to detect relatively low levels of alteration which cannot be discerned by biochemical or physiopathological methods. The objective of this investigation was to explore the feasibility of using fluorescent probes as a sensitive technique for detecting effects produced by the action of neurotoxic chemicals.

OBJECTIVES

1. To examine if selected neurotoxic chemicals induce conformational changes in nerve cells or their components <u>in vivo</u>, using fluorescent probes.
2. To establish the usefulness of fluorescent probes as a tool for predicting neurotoxicological hazards.

SECTION 2

CONCLUSIONS

The present investigation was undertaken to determine whether DDT, a neurotoxic pesticide, produces conformational changes in nerve membranes and if these changes can be detected by fluorescent probes. It has been shown that DDT alters ion permeability in nerve membranes.[5] Hilton et al.[3] and Hilton and O'Brien[4] have postulated that the mechanism by which DDT alters nerve membrane permeability involves modification of the membrane structure. It may be postulated that DDT, on account of its high lipid solubility, may expand cellular membranes leading to an alteration in their structure as has been reported for certain nonspecific lipid-soluble drugs.[6]

In the present study, using brain synaptosomes as a model system, we have demonstrated that any structural or conformational changes produced by DDT can not be detected by fluorescent probe technique suggesting that the technique is not a sensitive indicator of DDT-induced toxicity. This evaluation of the fluorescent probes is based on the assumption that the pesticide at the concentrations tested did induce changes in the structure of the synaptosomes.

SECTION 3

MATERIALS AND METHODS

Preparation of Brain Subcellular Fractions

Brain synaptosomes were prepared from male Sprague-Dawley rats fed *ad libitum* and weighing 200-250g. The animals were sacrificed by decapitation, the brain was excised and placed in cold (4°C) homogenizing medium. Rat brain synaptosomes were prepared according to the method of Gray and Whittaker.[14] The brain tissue was disrupted by homogenization in 0.32 M sucrose. The crude mitochondrial fraction was separated from the cell debris, red blood cells, nuclei and soluble fraction by means of differential centrifugation. Synaptosomes, free mitochondria, and myelin were then separated by means of density-gradient centrifugation. The scheme for the isolation of various subcellular fractions is outlined in Figures 1 and 2.

The protein content of synaptosomes was determined according to the method of Lowry *et al*.[15]

Fluorescence Measurements

Fluorescence measurements were made with an Aminco-Keirs spectrophotofluorometer (American Instruments Company) using an X-Y recorder. The light source was a Xenon lamp. Measurements were made at 25°C in a temperature-controlled cell holder connected to a circulating water bath. Aliquots of synaptosomal preparations were resuspended in 0.32 M sucrose

in 15 mM Tris-HCl buffer (pH 7.4) before making fluorescence measurements. The final synaptosome concentration was approximately 75 μg/ml unless indicated otherwise. An aliquot of ANS (8-anilino-1-naphthalene sulfonic acid) stock solution was then added to give a final probe concentration of 3.2×10^{-5}M, unless indicated otherwise. The fluorescence measurements were carried out at wavelengths corresponding to excitation and emission maxima determined by analysis of the spectrum of ANS bound to the synaptosomes.

ANS was obtained from Eastman Kodak and was recrystallized twice from boiling water before use.

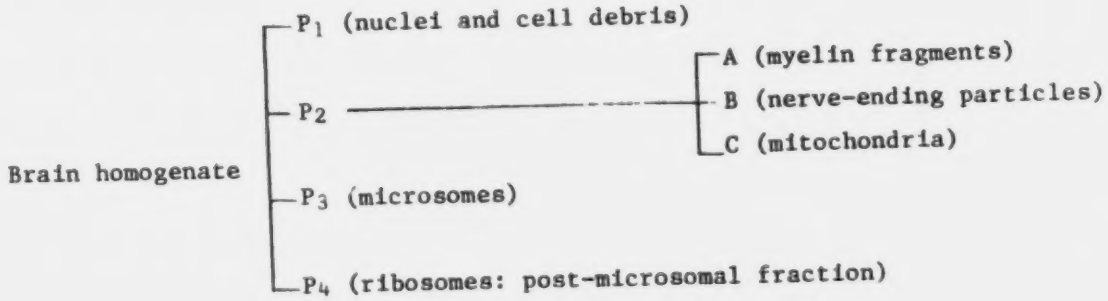

Figure 1. Nomenclature used in describing subcellular fractions.

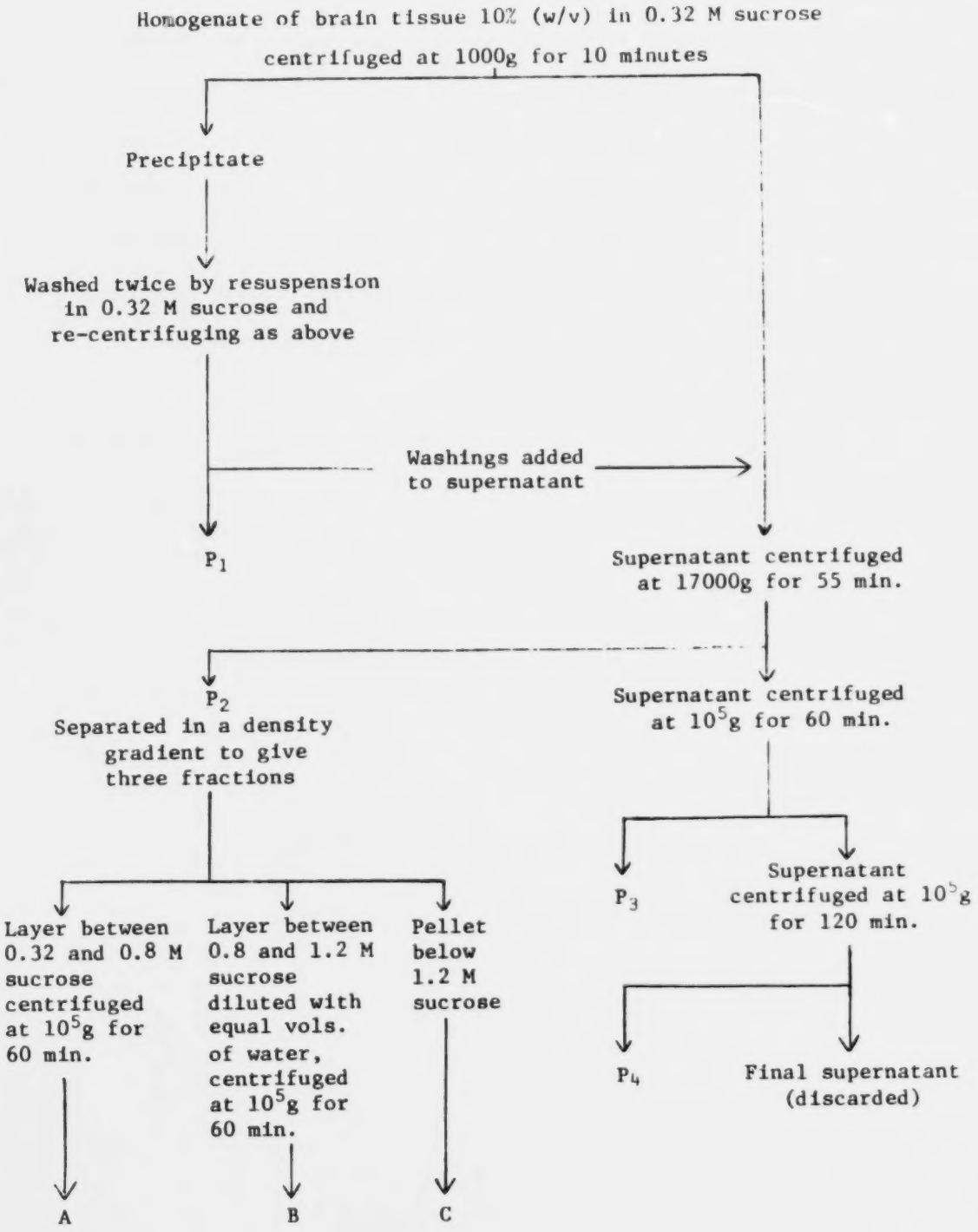
Homogenate of brain tissue 10% (w/v) in 0.32 M sucrose
centrifuged at 1000g for 10 minutes
Precipitate
Washed twice by resuspension in 0.32 M sucrose and re-centrifuging as above
Washings added to supernatant
P1
Supernatant centrifuged at 17000g for 55 min.
P2
Separated in a density gradient to give three fractions
Supernatant centrifuged at 10^5g for 60 min.
P3
Supernatant centrifuged at 10^5g for 120 min.
P4
Final supernatant (discarded)
Layer between 0.32 and 0.8 M sucrose centrifuged at 10^5g for 60 min.
Layer between 0.8 and 1.2 M sucrose diluted with equal vols. of water, centrifuged at 10^5g for 60 min.
Pellet below 1.2 M sucrose
A
B
C

Figure 2. Scheme summarizing preparation of subcellular fractions.

SECTION 4

EXPERIMENTAL PROCEDURES

Method of Tissue or Cell Treatment

Since absorption, distribution, excretion, and metabolism of a chemical determine its toxicity to the animal, it is desirable to expose the animal to the test chemical *in vivo* followed by *in vitro* testing to determine if the chemical produces any effect. The rats were exposed to the toxic substance, following which suitable tissues were removed and examined for possible conformational changes.

Selection of Cell and/or Cell Components Examined for Possible Conformational Changes

The techniques for measuring conformational changes using fluorescent probes, reproducibly and without complicated instrumentation requires that the biological material be in suspension. This precludes the use of brain slices or axons. In the present studies, which were designed to examine an overall effect of a given chemical on cell structure, it was desirable to use an isolated intact cell system in order to detect the possible conformational changes occurring in the whole nerve cell as well as its components. However, since techniques for the preparation of intact nerve cells are not currently available, we used nerve cell synaptosomes (presynaptic nerve-ending), as the test system because this fraction represents a well-characterized and functionally important part of the

neuron. There is evidence to show that synaptosomes retain most of the metabolic and membrane properties of the nerve ending in situ. As Whittaker[16] has pointed out, synaptosomes can be regarded as miniature, non-nucleated neurons, or even as model cells.

Type of Fluorescent Probe Used

Both intrinsic and extrinsic fluorescent probes were used in these studies to detect any possible conformational changes. In these studies, a fluorochrome, 8-anilino-1-naphthalene sulfonic acid (ANS) was used as an extrinsic probe since it has been most commonly used in detecting conformational changes in membranes. Anesthetics and certain drugs which are known to act on the cell membrane have been found to cause a change in the ANS fluorescence in the presence of microsomal, erythrocyte and mitochondrial membranes.[18,19,20,21,22] Spero et al.[7] reported that tetrodotoxin, a neurotoxin, reduced the ANS fluorescence increase in guinea pig brain synaptosomes.

Fluorescence parameters including (i) fluorescence intensity, (ii) fluorescence spectra, and (iii) energy transfer from intrinsic membrane chromophores to ANS were measured to determine if any conformational changes in the test system had occurred.

SECTION 5

RESULTS

Interaction of ANS with Rat Brain Synaptosomes

Addition of synaptosomes to a dilute solution of ANS resulted in a marked enhancement of the fluorescence measured at 490 nm, using light of 390 nm wavelength for excitation. The enhancement of ANS fluorescence due to synaptosomes was accompanied by a shift of the emission maximum from 520 to 490 nm wavelength. Since ANS does not fluoresce in aqueous solution, the appearance of fluorescence on addition of synaptosomes indicates an interaction of the dye molecules with synaptosomes. An enhancement of ANS fluorescence and a shift of the maximum emission to shorter wavelengths is characteristic of a change in ANS environment from one of high to low dielectric constant. On the basis of the above observations, it is reasonable to assume that ANS is bound to hydrophobic phase when this compound interacts with synaptosomes.

Figure 3 indicates a titration of 6×10^{-5}M ANS with brain synaptosomes (390 nm excitation, 490 nm measurement). Figure 4 illustrates the titration of 75 µg/ml of synaptosomal protein/ml with ANS.

Effect of an Acute Dose of DDT on Synaptosome Fluorescence

In these experiments the effect of DDT was studied following administration of a single dose which produced overt neurological symptoms in the animals. Sprague-Dawley rats (250-300g) were fasted overnight before being

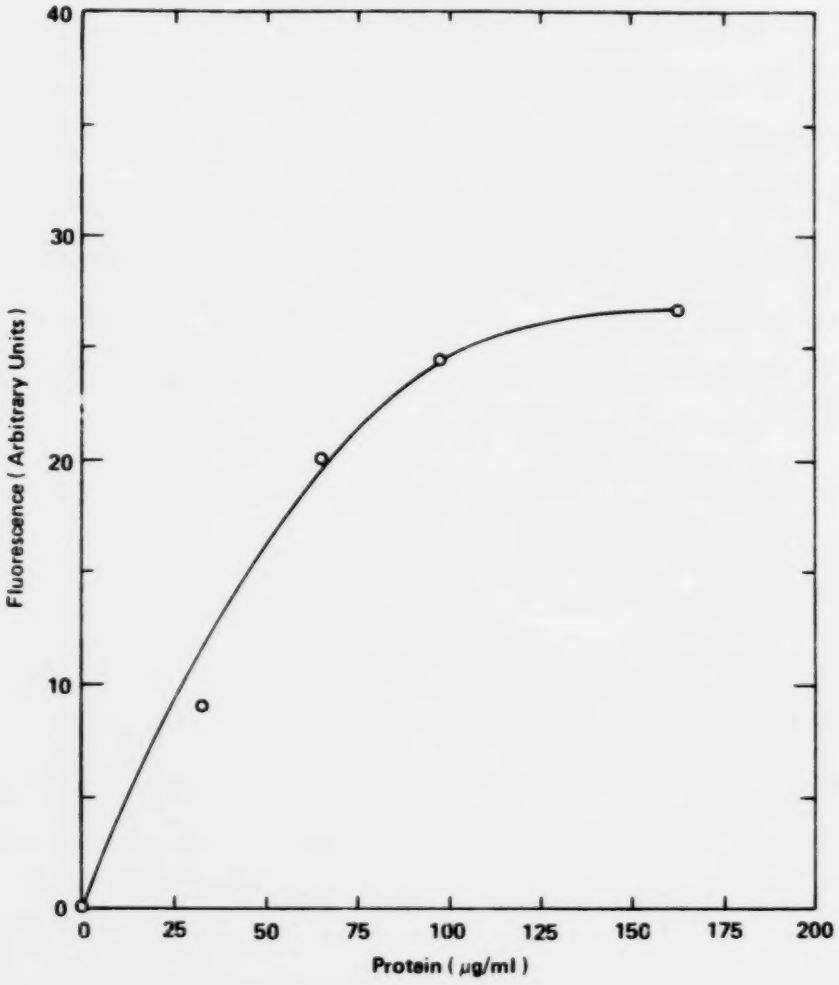

Figure 3. Titration of ANS (6×10^{-5} M) with brain synaptosomes

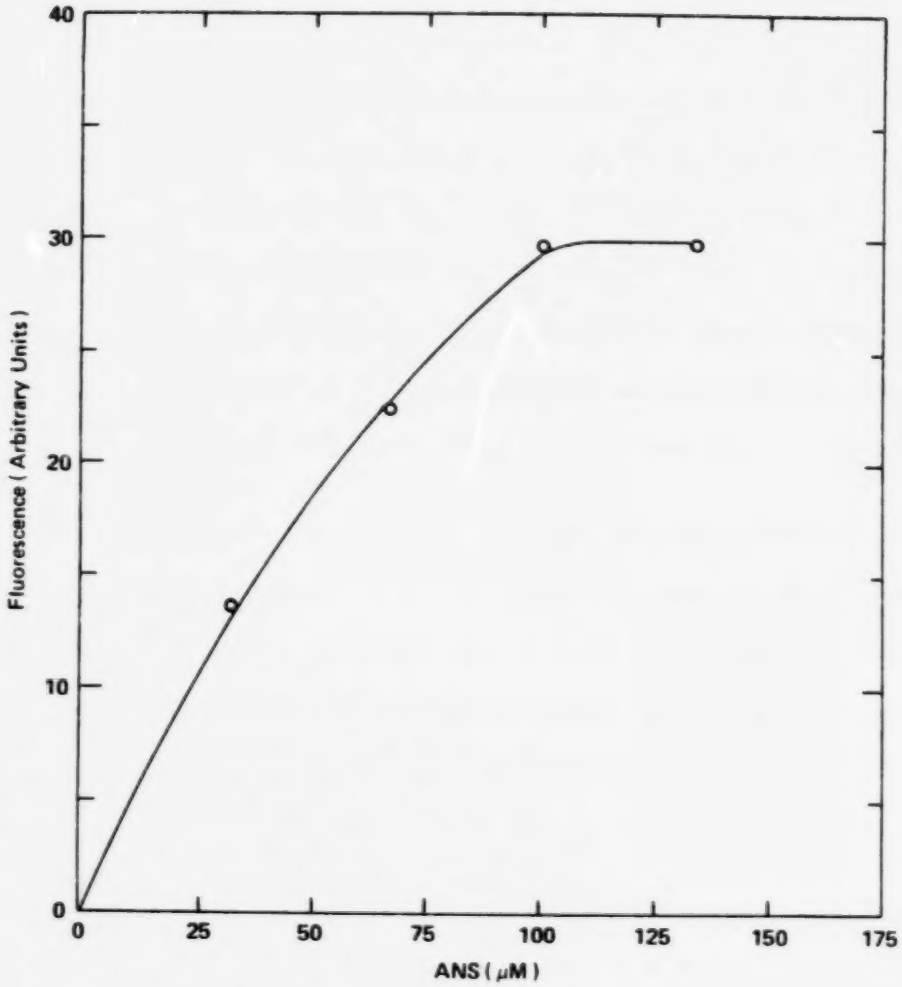

Figure 4. Titration of brain synaptosomes (75 μg protein/ml) with ANS

given high purity DDT in corn oil by oral intubation at a rate of 150 mg/kg body weight. Control animals were given corn oil. The treated animals showed tremors within 4 hours. They were then sacrificed by decapitation, the brain was excised and the synaptosomes were prepared as described earlier.

Aliquots of synaptosomal preparation were resuspended in 0.32 M sucrose in 15 mM Tris-HCl buffer (pH 7.4). The final membrane concentration was approximately 75 µg of protein/ml. An aliquot of ANS stock solution was then added to give a final probe concentration of 3.2×10^{-5}M and fluorescence measurements were performed in an Aminco-Keirs spectrofluorometer. The excitation and emission wavelengths were 390 and 490 nm, respectively. The emission spectrum of membrane-bound ANS was also measured.

Fluorescence Intensity and Spectra

The results showed that the fluorescence intensity and spectra of the ANS bound to synaptosomes from control and DDT-treated rats did not differ. DDT treatment also caused no change in the fluorescence intensity of ANS bound to brain mitochondria. These findings represent the results of three separate experiments.

Effect of Energy Transfer from Intrinsic Membrane Chromophores to ANS

The excitation spectrum of ANS overlaps the emission spectrum of tryptophan and tyrosine. One might expect a measurable transfer of excitation energy from membrane tryptophane and tyrosine to ANS molecules inserted in close proximity to these amino acids. Therefore, in the presence of ANS, excitation of protein fluorescence by 280 nm light produces energy transfer from tryptophan emission to bound ANS resulting in ANS fluorescence with an

emission peak at about 490 nm. It has been shown that energy transfer is sensitive to even a small perturbation in membrane conformation.[11]

To determine if DDT treatment had an effect on energy transfer, DDT-exposed or control membrane suspensions (protein content 75 µg/ml) were placed in cuvettes and the intrinsic protein fluorescence peak (340 nm) was read following excitation with 280 nm light. ANS was then added to the cuvette and the emission was recorded following excitation at 280 nm. Treatment with DDT did not cause a change in ANS emission resulting from excitation at 280 nm, suggesting that the pesticide does not affect the transfer of tryptophan emission energy to ANS.

Effect of DDT on Calcium-dependent Enhancement in Fluorescence

The action of DDT on synaptosome-bound ANS fluorescence was further evaluated by examining the effect of the pesticide on a calcium-dependent increase in ANS fluorescence. Divalent cations including Ca^{2+} cause an increase in the red cell membrane-bound ANS.[20,23] It is likely that if DDT induces any conformational changes in synaptosomes, such changes may modify the ANS-fluorescence in response to Ca^{2+}. We observed that Ca^{2+} enhanced the fluorescence intensity of synaptosome-bound ANS by about 60%. When synaptosomes from DDT-treated rats were incubated with Ca^{2+}, no change in fluorescence intensity or quantum yield was observed as compared to untreated synaptosomes also incubated with Ca^{2+}.

Effect of DDT on Synaptosomal Protein (Intrinsic) Fluorescence

The intrinsic chromophores of the proteins in the membrane originate from the amino acids, tyrosine and tryptophan.[24] A change in fluorescence intensity or a shift in the position of the peak of intrinsic fluorescence

can be used as a measure of conformational changes resulting from treatment with membrane perturbants. The intrinsic fluorescence technique has been used to study the interaction of anesthetics and hormones with erythrocyte membranes and proteins.[25,26]

For measurement of intrinsic protein fluorescence, the fluorescence emission was scanned from 300-400 nm following excitation with 280 nm light. The peak height, which was usually at 340 nm in control synaptosomes was recorded. Our results showed that treatment with DDT did not alter the intensity or emission spectrum of the native protein fluorescence.

Effect of Repeated Doses of DDT on Synaptosomes Fluorescence

The above studies showed that a single dose of DDT which produced overt neurological symptoms in rats, did not induce conformational changes in brain synaptosomes. Additional studies were undertaken to examine whether or not administration of repeated sublethal doses of the pesticide to rats over an extended period induced conformational changes in brain synaptosomes.

In these studies, two groups of rats (200-250 gm) were given DDT at two different dosage levels daily for eight consecutive days. One group of animals was administered the pesticide daily in corn oil by oral intubation at a rate of 25 mg/kg body weight. These experiments were repeated with the second group using a dose of 50 mg/kg. Control animals were given corn oil. None of the animals in either of the treated groups showed obvious symptoms of toxicity at the end of the treatment period. They were then sacrificed by decapitation, the brain was excised and the synaptosomes were prepared according to the method of Gray and Whittaker.[14] The effect of DDT on synaptosomes as measured by extrinsic and intrinsic fluorescence was determined as described in single-dose studies.

The results showed that like the single dose of DDT, administration of repeated doses of the pesticide at the levels used in the study did not induce any conformational changes in brain synaptosomes as measured by ANS fluorescence (intensity, emission spectrum and energy transfer). DDT treatment also did not alter the intensity or emission spectrum of intrinsic fluorescence.

Effect of Diphenylhydantoic on Synaptosomal Membranes Exposed to the Drug in vitro

Diphenylhydantoic (dilantin®) is an important drug used to protect epileptic patients against seizure. It has been suggested that Dilantin® interacts with plasma membranes and exerts a stabilizing effect on all excitable cell membranes.[27,28] It was therefore of interest to determine if the drug induces conformational changes in synaptosomal membranes as measured by intrinsic and ANS fluorescence.

In these studies, the in vitro effect of Dilantin® on the fluorescence of ANS bound to synaptosomes isolated from untreated rats was examined. The synaptosomes were incubated with the sodium salt of diphenylhydantoin (10^{-6} to 10^{-4} M) for fifteen minutes. An aliquot of ANS solution was then added to give a final probe concentration of 5×10^{-5}M and fluorescence measurements were made as described above. We observed that the Dilantin® up to a concentration of 10^{-4}M did not cause any change in the fluorescence intensity or spectrum of ANS. The chemicals also showed no effect on intrinsic fluorescence.

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